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A STUDY OF "IMMEDIATE" SENSITIZATION BY ADSORPTION OF ANTIGENS AND ANTIBODIES *IN VITRO*

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INTRODUCTION

Research under the purview of Contract ONR N6 225(46) has two general directions. (1) the study of the mode of action of protein toxins, and (2) the study of the mechanism of anaphylaxis, particularly with respect to the process of sensitization. Under each heading we are prosecuting several distinct problems which have been selected both for the theoretical implications in immunochemistry and biochemistry as well as their practical applications to human ecology and disease.

The following is an outline of the specific problems pursued this year:

I. TOXINS

A. Tetanus: In previous reports there have been presented detailed accounts of the isolation and characterization of the non-spasmogenic factor in tetanus toxin which produces an increased discharge of miniature end plate potentials. This material had been isolated in small quantities as a single antigen, and sufficient material was available to carry out determinations of molecular weight and a tentative amino acid analysis. This year we concentrated our efforts at improving the yield so that sufficient material could be available for physical-chemical and biochemical characterization. Ultracentrifugal analyses of the best fraction available last year showed the lethal fraction to have a Svedberg coefficient of 7 whereas the most potent MEPP-fraction was associated with 4*S* particles. This year, further preparative chromatographic separations were made on the MEPP-rich starting material (Fraction IV). Chromatography on Sephadex G-200 gave 3 major peaks. The yields of repeated runs were separately pooled on the basis of elution diagrams and each pool was concentrated by pressure-dialysis. No appreciable MEPP activity was found in the first 2 peaks; it appeared to be concentrated in Peak III, which had no lethal activity. This peak had only one antigenic determinant and gave a Svedberg coefficient of 2; but all of the lethal toxicity of the parent was quantitatively recovered in Peaks I and II which contained 5*S* and 4*S* molecules, respectively. The MEPP material accounted for only 7% of the total protein of the starting material.

B. Streptolysin: Studies on the mode of cardiotoxic action of Streptolysin O have shown that there is a double insult to the heart, one of these is reversible, and is due largely to the output of acetylcholine, while the other appears to be more severe and seems to result from profound changes in cellular economy as exhibited in recent studies of the release of K^{42}.

In appropriate concentrations the activated toxin causes a transient decrease in the rate and amplitude of contraction of the isolated atrium accompanied by an increase in the rate of repolarization of the atrial action potential. The severity of the effects depend upon the dose of toxin, and their magnitude decreases with succeeding challenges, suggesting that Streptolysin O may exert its action on the isolated atria through the release of an intermediate from tissue stores. This year, detailed studies of the mode of

cardiotoxic action suggested that the chronotropic and inotropic changes exhibited by the isolated atria when they were challenged either in the presence of a blocking agent (atropine) or in the presence of anti-cholinesterases (eserine or DFP) were due to the release of acetylcholine. The electrical changes in single atrial cells - increased resting potential, accelerated repolarization, as well as the generally negative results observed during ventricular impalements - were consistent with the acetylcholine hypothesis. In spite of the presence of powerful tissue cholinesterases sufficient quantities of material were obtained by incubating large numbers of challenged atria with toxin to render pharmacological identification of Ach unequivocal.

C. Sea Urchin Toxin: The crude pedicellarial toxin of *Tripneustes gratilla* obtained from 1,000 specimens in 1966 and 2,000 specimens during 1967 was variously fractionated, and the fractions further purified by chromatography on Sephadex. The enzymological properties of these materials were studied with respect to toxin concentration, substrate concentration, and temperature. The kinetics of the reaction system with respect to whole plasma and to its pseudoglobulin fractions are of a complex order owing to (1) "natural" kinin formation of the substrate, (2) to the inactivation of the reaction product (and of synthetic bradykinin) by the toxin, and (3) to the existence of more than one enzyme in the "purified" material. Plots of first order velocity constants against the square of substrate concentration were linear suggesting the existence of substrate "modifiers". The dependence of reaction velocity on enzyme concentration was not of the classical type. The temperature optimum of the system was 26°C. A study of the reactions of various Cohn fractions with more purified enzymes suggested that the substrate furnishing the kinin activity on guinea pig tissues was α_2-globulin and conclusive proof was obtained in studies with immunoelectrophoretically pure α_2-macroglobulin. Parallel assays on guinea pig and rat tissues suggested that the substrate responsible for the oxytocic effect in the rat, on the other hand, is a β-globulin. Rabbit antibodies, produced in response to inoculations of formalin-toxoids in Freund's adjuvant, gave normal quantitative precipitin curves with "active" antigens (or toxoids) and they fixed complement. Immunoelectrophoresis showed 2 major antigens, the more immunogenic being concentrated in the 2/3 SAS fraction while the other was present among the 1/3 SAS materials. Biologically, the antisera showed quantitative antitoxic action in the usual protection and neutralization tests in whole animals as well as on isolated tissues. Toxin-induced inhibition of active Na transport in the toad bladder was also neutralized by the antitoxin.

II. ANAPHYLAXIS

1. Ragweed: Evidence was obtained that rabbit anti-ragweed conferred passive sensitivity on cardiac and intestinal tissues of the guinea pig. The degree of sensitization, estimated by histamine release, was shown to depend on the [AB] in the bulk phase. For a constant degree of sensitization the response to the whole antigen could be quantitatively inhibited by the presence of a dialyzable ragweed hapten during challenge with the non-dialyzable antigen.

2. Penicillin: Positive Schultz-Dale reactions were obtained to penicilloyl poly-lysine in isolated guinea pig tissues sensitized with rabbit antibodies to penicilloyl-rabbit serum albumin (anti-Pen RSA)

and to penicilloyl Rabbit γ-globulin (anti-Pen RγG). The reaction could be quantitatively blocked by ε-aminocaproic acid and by the penicilloyl derivative of the condensation product between DAB (1, 4-diaminobutane) and fluorescein isothyocyanate. The eventual aim of these studies is to set conditions for the determination of the kinetic behavior, binding affinities, and heterogeneity constants of "allergic" antibodies to penicillin by measurements of fluorescence polarization.

3. Fc Fragments: Quantitative reversed passive sensitization with F_c fragments of rabbit γ-globulin was demonstrated in the *in vitro*-sensitized heart challenged with ovine antiserum prepared against these fragments. The concentration of Fc fragments required to produce 50% sensitization was found to be 0.07 mg/ml and the liminal concentration was 3×10^{-4} mg/ml.

The following Progress Reports contain detailed accounts of work on Sea Urchin Toxin and on Ragweed. It is expected that similar details for Streptolysin and Tetanus will be presented shortly as soon as the experiments now in progress have been completed.

PART I

SEA URCHIN TOXIN

INTRODUCTION

The present report deals principally with certain aspects of the physical-chemical, pharmacological, and immunochemical characteristics of the protein venom of the sea urchin, *Tripneustes gratilla* as well as with accomplishments in two specific areas in which its point of attack was experimentally restricted: its action as an enzyme, and its effectiveness in producing changes in active transport.

I. EXPLORATION

During the summer of 1966 Dr. Feigen and Dr. Terres made several exploratory field trips to the Pacific coast between Half-Moon Bay, on the north, to Santa Barbara in the South. The northern collections, made at Half-Moon Bay, Pigeon Point, and Pacific Grove yielded only *S. purpuratus* and *S. franciscanus*. Fifty specimens of each were collected by diving at depths of 10-20 feet at Pigeon Point; thirty specimens of *S. purpuratus* were collected off the rocks at low tide at Half-Moon Bay, on the extreme seaward limit; virtually none was got at Pacific Grove because of the great number of collectors using these materials in that area; and fifty specimens of each were obtained in deep water around Santa Barbara, as well as a few specimens of *Lytechinus anamesus*. We are indebted to the Director of the Marine Laboratory at U.C.S.B., Professor John E. Cushing, for this courtesy. Neither *purpuratus* nor *franciscanus* was found to have extractable venom. *Lytechinus anamesus* was tentatively identified, on the basis of pharmacological tests, as being acetylcholine or a kindred cholinergic substance.

Through the courtesy of Professor T. A. Rogers, Director of the Pacific Bio-Medical Research Institute of the University of Hawaii, Dr. Feigen and Dr. Terres spent three weeks of September 1966 at the University Aquarium in Honolulu. That laboratory served as a base of operations for collecting, processing and concentrating venom. With the aid of Mr. Roger Pfeffer, five diving expeditions beyond the reef off Waikiki Beach netted 1050 specimens which were processed in the Aquarium's laboratory. The crude protein was 1.7 grams of material having a potency equivalent to 333 LD_{50} doses/mg when assayed on a population of 20 gram mice.

II. CHEMICAL

Preparation of Crude Pedicellarial Proteins of *T. gratilla*

The method of extraction and preparation is given in the following protocol for Collection IV.

Collection IV was made with Scuba equipment at a depth of 30 feet from the underwater canyon of Coral Reef. The coordinates were the intersection of a line drawn between Diamond Head and Kewalo Basin, and that passed at right angles from the Royal Hawaiian Hotel - at approximately the 5-fathom contour.

The number of specimens collected was 359. The specimens were processed in batches of 16 by being placed in a frame and washed with a strong sea water spray. Larger material was retained by a fiber-glass screen and the pedicellariae were caught in a stainless steel sieve having interstices of 125 microns. The contents of the screen were transferred to 500 ml cylinders and the pedicellariae separated from the detritus by elutriation with sea water. The elutriated pedicellariae were washed with sea water until the washings became colorless.

1. The residues of pedicellariae were collected and centrifuged for 30 minutes at 500 rpm. The yield was 55 ml of a gummy pack. The total yield was homogenized in a Waring Blendor at 4°C with 260 ml of distilled water.

2. The homogenate was centrifuged as above. The first supernate was removed and re-centrifuged at 10^4 rpm for 2 hours at 4°C.

3. The clear supernate was dialyzed against phosphate buffer, (μ = 1/60) for 20 hours at 2°C.

4. The residue of (2) was re-extracted with 200 ml of ice-cold water, centrifuged and dialyzed as in (3)

5. The non-dialyzable extracts were dried from the frozen state. The dialyzates were frozen and shipped to Stanford, where they were pooled, redialyzed, lyophilized, and tested.

6. The yield of non-dialyzable material from 359 animals for the first and second extractions is given in Table I.

7. The yields as well as certain physical-chemical and pharmacological properties of both the dialyzable and non-dialyzable crude extracts of the 1966 toxin are given in Table II.

Fractionation of Crude Toxin on Sephadex

Our previous studies had established that the most toxic fraction which could be obtained by separation with ammonium sulfate was the one precipitating in the presence of 65% salt saturation. In the present case a fractionation was attempted (by Dr. C. B. Alender) on Sephadex with the entire yield of whole extract of 65 pedicellariae. The method of preparation is exhibited in the flow sheet of Table III.

Three peaks were obtained and each fraction was precipitated with the solid ammonium sulfate, dialyzed, and Fractions I and II were tested. The toxicity of Fraction III was so low as to be beyond the stocks of the material available and at 735 LD_{50}/mg was as active a preparation as had been obtained in our experience. Some of the characteristics of these fractions are given in Table IV.

Isolation of the Test Proteins of *S. purpuratus* by Ammonium Sulfate Fractionation

This study was undertaken to characterize the various protein components of the tests of *S. purpuratus* for use in subsequent investigations of immunochemical kinship. The present separations were made on the sea water homogenates of 50 eviscerated specimens obtained in Santa Barbara. Ammonium sulfate fractions were prepared in the usual way be sequential precipitation with salt and removal of material at each interval. As indicated in Table V, the major bulk of precipitate was obtained in the presence of 50% saturation.

The materials to be used for subsequent antigenic analysis were characterized by analytical ultracentrifugation, as these fractions corresponded in physical properties to the toxins of *T. gratilla*. From Table V it is evident that the fraction precipitating at 2/3 saturation contained most of the components of the crude material. The number of components was greatly reduced as the fractionation proceeded. None of the materials tested was toxic to mice and none had an effect on the isolated tissues of the guinea pig.

III. PHARMACOLOGICAL

A detailed pharmacological analysis of the action of sea urchin toxin has just been published from this laboratory by Feigen, Sanz, and Alender, [*Toxicon, 4:*161-175, (1966)]. Some of the pertinent findings are summarized in the succeeding paragraphs.

Direct tests with crude non-dialyzable preparations of sea urchin toxin as well as with the fractions prepared by ammonium sulfate precipitation induced prolonged contractions of the isolated guinea pig's ileum in proportion to the dose used. The response was not blocked with atropine, only partially blocked with *Pyribenzamine,* blocked with various degrees with D-bromolysergic acid and *Mellaril,* and completely blocked with phenyl-butazone.

A. Direct Actions: Since the direct exposure of gut strips to toxin preparations often changed the tissues' sensitivity to histamine, studies of the dose-response characteristics had to be made by exposing each strip only once to a given concentration of the toxin. The dose-response curve, obtained by pooling the data over a range of concentrations, obeyed the usual logistic function, and this could be employed reliably to assess changes in potency resulting from various physical and chemical treatments. Gentle heating of toxin reduced the magnitude and the slope of the dose-response curve, showing that the incubation of toxin for a constant time was inversely dependent on its concentration; carbon treatment decreased the median effective dose but increased the heterogeneity of the response, and treatment with ammonium sulfate as seen in Table VI, concentrated most of the activity of the starting material in the fraction precipitating in the presence of 65 per cent saturated salt solution. The toxin was shown to release dialyzable pharmacologically active agents from ileal, pulmonary, and cardiac tissues of the guinea pig as well as from colonic and pulmonary preparations of the rat.

B. Release of Active Materials: Preliminary tests excluded acetylcholine but suggested that histamine, as well as several other substances, could be released by the reaction. Subsequent specific chemical analyses confirmed the presence of histamine.

The release of histamine by the isolated tissues was shown to be quantitatively dependent upon the toxin concentration (Table VII), and the temperature (Table VIII). Although none of the fractions was as potent as the parent material, the preparation obtained in the presence of 65 per cent saturated ammonium sulfate was the most potent histamine releaser among the fractions, being only slightly less active than the starting material.

IV. IMMUNOCHEMICAL

Studies on the immunochemical properties of sea urchin toxin were initiated by the inoculation of 5 mg amounts of sea urchin toxin preparations, mixed with complete Freund's adjuvant intramuscularly into rabbits. The injection sites rapidly ulcerated and the animals shortly died from the toxic effects of the preparations. Immunization was then made with a crude preparation of [SUT(64)] which had been toxoided with 0.01% formaldehyde. Intramuscular injections were made with 1 mg amounts of [SUT(64)] in complete Freund's adjuvant and the animals showed no ill effects. Two weeks later the rabbits were bled and the sera showed positive ring tests when tested against [SUT(61)]. Immunoelectrophoresis showed that a single precipitin line was formed between [SUT(61)] and the antiserum prepared against [SUT(64)]. The mobility of the antigenic determinant had the character of that of serum α-globulin, migrating towards the anode from the central agar well. Complement fixation tests, performed on the toxin-antitoxin system, showed that [SUT(61)] fixed complement at a concentration of 0.01 mg/ml when tested with the antiserum at a 1/4 dilution.

In finding that antibodies could be produced against the toxoid of [SUT(64)], further studies were made in an attempt to isolate the responsible antigen from the crude SUT Preparations. SUT(64-2) was fractionated at 33, 65 and 100% saturated ammonium sulfate and the dialyzed products were toxoided and injected into rabbits in the same manner as in the previous experiments; crude SUT-66 was also injected in this way.

The respective sera were harvested, and each homologous toxoid and anti-toxoid serum showed positive ring tests. Moreover, the crude toxin [SUT-1966] showed positive ring tests with the sera prepared against 33, 65, and 100% saturated ammonium sulfate fractions of 64-2 as well as against its homologous antiserum. Immunoelectrophoresis experiments using crude SUT-66 against its homologous antiserum and the ammonium sulfate fractions of 64-2 showed that a single line was formed in each instance, the character of the line being the same as that seen in SUT-1961.

Further experiments, using sea urchin protein from *S. purpuratus*, showed that antiserum produced against the 100% as fraction of [SUT(64-2)] cross-reacted with the *S. purpuratus* protein.

V. BIOCHEMICAL

On the basis of the preceding pharmacological work it was inferred that several substances were released in addition to histamine. On the basis of the results given in Table IX it was suspected that serotonin as well as a dialyzable kinin might be released.

A test of the ability of sea urchin toxin to act as an enzyme was made with beef serum, bovine serum albumin, and bovine γ-globulin as substrates. Although positive reactions were obtained with the dialyzates obtained from the beef serum system, the other two substrates yielded no active dialyzable material. Tests were next made to determine whether the behavior of the system was normal with respect to the influence of substrate concentration on velocity by estimating first order time-courses for each category of substrate concentration. The results given in Table X show that the first order velocity constants form a sigmoidal curve against concentration.

The existence of a sigmoidal function in that regard suggested that the reagents were probably both impure. Since plasma kinins - bradykinin in particular - are formed from the enzymatic attack of the enzyme upon α_2 -globulins it was considered useful to test several plasma fractions containing varying concentrations of α_2 and β-globulins. Cohn Fractions III-0 and IV-1 were obtained through the courtesy of Mr. Kingdon Lou of the Hyland Laboratories in Los Angeles. They were extracted with saline, centrifuged, and adjusted to a protein concentration of 5 mg/ml. Separate ampoules of the material were frozen and thawed only once before experimental use. Fraction III-0 contained 5% α-globulin and 84% β-globulin while Fraction IV-1 contained 89% α-globulin and 10% β-globulin.

Comparative experiments were first made to determine the substrate potencies of III-0, IV-1 and normal beef serum at 2 enzyme concentrations of [SUT(64-2) 65 as]. Tests were made on the guinea pig ileum and rat uterus. In general more Bradykinin-like activity was elaborated by Fraction III-0 than by Fraction IV-1, with respect to the guinea pig's ileum although it was about the same for rat uterus (Table XI).

The effect of enzyme concentration on kinin production by the attack of SUT 65 as on III-0 was studied with respect to the activities of guinea pig ileum and rat uterus. The results given in Table XII show that SUT is a much more effective enzyme for a substrate in the mixture which is effective on guinea pig ileum. The effect of varying the substrate concentration (III-0) is shown in Table XIII.

A comparison was next made between III-0 and IV-1 at such substrate and enzyme concentration at which the respective reactions would be maximal in order to determine whether there would be differences in output of kinin. Table XIV shows that these conditions were achieved and that the mean output of kinin with respect to the guinea pig ileum test was about 4 times greater in the case of III-0 than of IV-1

Finally, the two impure substrate preparations were tested in such a way that there was a constant amount of α_2- and β-globulin, by adjusting the concentration with respect to the percentage composition of the two individuals, as given earlier. Table XV shows that when β-globulin was constant the same amount of kinin was produced with respect to guinea pig ileum. In the case of the rat uterus none was produced by Fraction III, leading to the conclusion that β-globulin is not a substrate for the production of the "rat" kinin but is useful in the production of "guinea pig" kinin. When α was constant more activity was evident in both cases but it was greater for both assays in the case of III-0 which had a higher α content.

VI. PHYSIOLOGICAL

Active Transport

The effect of the most active fraction of purified sea urchin toxin [SUT(64-2) 65 as] on the sodium pump was tested on the toad (*Bufo marinus*) urinary bladder. The bladder was mounted as a diaphragm in the Ussing apparatus, and the transmembrane and the short circuit currents were measured before and after the addition of the toxin. To date the following points have been established:

1. The addition of toxin to the serosal side of the membrane results in an immediate drop in both the transmembrane potential and the short circuit current. The magnitude of the drop is approximately the same in both cases, i.e. about 50%. The effect is transient with recovery occurring within 10 to 20 minutes.

2. Addition of the toxin to the mucosal surface has no effect.

3. This effect can be abolished by heat-denaturation of the toxin (1 hr. at 55°C)

4. This effect is not potentiated by preincubation of the toxin with normal rabbit serum.

5. This effect is not due to the release of acetylcholine, serotonin, or histamine. These drugs, when tested on the urinary bladder, did not have a noticeable effect.

TABLE I

Yield of Non-Dialyzable Material During First and Second Extractions

	Category	Volume ml	Net Weight g	Total First Yield (less phosphate) g
Non-dialyzable material	1st Extract	225	0.964	1.11
	2nd Extract	200	0.476	

TABLE II

Summary of Characterization of Sea Urchin Toxin Preparation Collection of September, 1966.

	Final Yields	
	Total	Per Specimen
Total Specimens	1050	
Total organic material	2.950 g	2.77 mg
Non dialyzable matter	1.200 g	1.14 g
Dialyzable matter	1.705 g	1.62 mg

CHARACTERIZATION

	Non-dialyzable	Dialyzable
Nitrogen		
Total N (per mg)	0.66	0.07
Precipitable N (per mg)	0.0456	none
Spectrophotometry		
Peaks	278	270 278 320
OD/mg.ml^{-1}	2.14	0.460 1.70
Ultracentrifugation		
S_{20}	2.2 7.5	none
Bioassay		
LD_{50}	0.151 µg/gm	400 µg/g
Guinea pig gut	1.60×10^{-3} mg/ml	0.50 mg/ml
Guinea pig heart	1.70×10^{-3} mg/ml	0.23 mg/ml
Rat uterus	not done	0.20 mg/ml

TABLE III

Sephadex Fractionation of Crude Sea Urchin Toxin

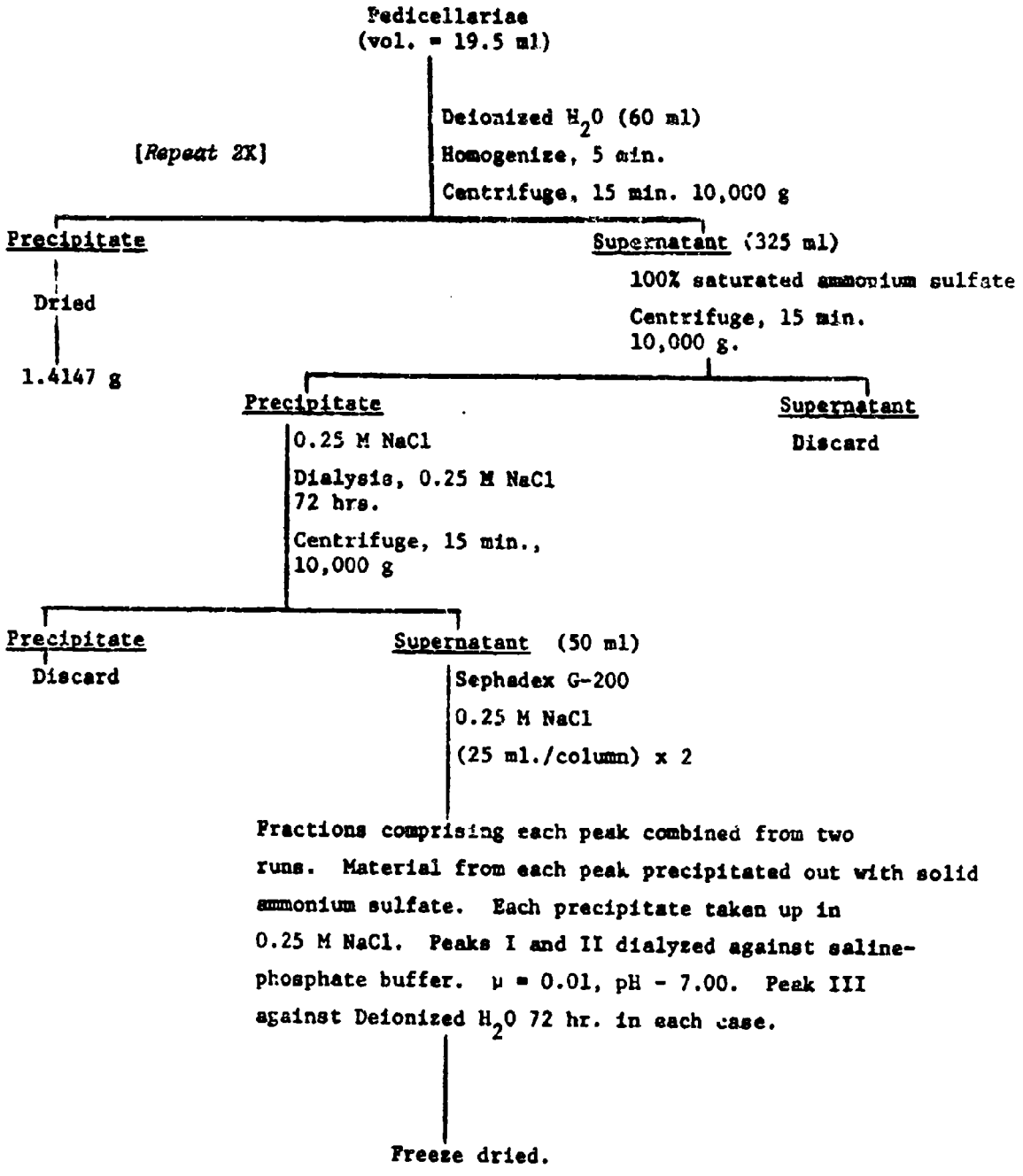


TABLE IV

Properties of Sephadex Fractions of Crude Sea Urchin Toxin

	Weight g	LD_{50}/mg	OD_{260} 1 mg/ml	OD_{280} 1 mg/ml
Starting Material	1.42	333		
Fr. I	0.106	<0.0035	0.48	0.42
Fr. II	0.072	735	0.55	0.69
Fr. III	0.036	not tested		

TABLE V

Material Balance of *S. purpuratus* Test Proteins

Precipitated with Ammonium Sulfate

Fraction SAS (%)	Total Protein Precipitated mg	Percentile Distribution of Protein Precipitated (%)	Sedimentation Constants S_{20}
0*	900		3, 21, 36
25	76.0	15.38	
33	97.0	19.64	
50	154.5	31.28	
67	61.0	12.35	3, 12, 23, 35
75	50.4	10.20	2, 25
100	55.0	11.40	
Total Recovery	439.9 mg		
Per Cent Recovery	55%		

*Starting Material

TABLE VI

Effect of Ammonium Sulfate Fractionation on Pharmacological Potency of Sea Urchin Toxin Assayed on Guinea Pig Ileum

Preparation	No. of Tissues	† SUT Conc. (mg N/ml)	Histamine Equivalent (moles/l)	Median Effective Dose of SUT (mg N/ml)	LD_{50} (mg N)
SUT(64)	2	1.86×10^{-4}	2.0×10^{-7}	6.8×10^{-4}	2.06×10^{-4}
	2	3.71×10^{-4}	2.4×10^{-7}		
	2	7.42×10^{-4}	3.6×10^{-7}		
	2	1.49×10^{-3}	6.4×10^{-7}		
	2	2.98×10^{-3}	8.0×10^{-7}		
SUT(64)33 *as*	2	8.78×10^{-4}	3.0×10^{-8}	1.5×10^{-3}	No toxicity
	3	1.94×10^{-3}	1.3×10^{-7}		
	2	2.91×10^{-3}	1.5×10^{-7}		
	4	3.54×10^{-3}	1.8×10^{-7}		
SUT(64)65 *as*	2	1.89×10^{-4}	8.0×10^{-8}	7.4×10^{-4}	3.5×10^{-4}
	2	3.76×10^{-4}	1.8×10^{-7}		
	2	7.73×10^{-4}	2.0×10^{-7}		
	2	1.55×10^{-3}	2.8×10^{-7}		
	2	2.34×10^{-3}	3.0×10^{-7}		
	3	3.89×10^{-3}	3.9×10^{-7}		

* *as*: % saturation of ammonium sulfate

† SUT: Sea Urchin Toxin

TABLE VII

Effect of Toxin* Concentration on the Release of Histamine From Guinea Pig Gut

Sea Urchin Toxin (mg N/ml)	Histamine† (µg/mg) Dry Tissue
2.68×10^{-4}	1.10×10^{-3}
5.40×10^{-4}	2.51×10^{-3}
1.34×10^{-3}	3.18×10^{-3}
2.68×10^{-3}	3.64×10^{-3}

* Sea Urchin Toxin [SUT(61)-C-64].

† Quantity released after incubation for 60 min at 37°C, estimated chemically.

TABLE VIII

Effect of Temperature on Histamine Released From Guinea Pig Lung by Sea Urchin Toxin*

Temperature (°C)	Histamine Release† µg/mg wet tissue
10	3.29×10^{-5}
21	3.51×10^{-5}
30	4.67×10^{-5}
37	6.87×10^{-5}

* incubated with sea urchin toxin [SUT(64)] (4.90×10^{-3} mg N) for 20 min.

† Chemical estimation

TABLE IX

Comparative Blockade of Dialyzable Kinin and Known Drugs With Various Antagonists

Conc. of Test Compound	Blocking Agent Type	mg/ml	Initial Reaction (% of maximum)	Reaction After Blocking Agent	% Block
Dialyzable Kinin 1.21 mg/ml	Atropine	0.714	29	26.60	8.0
	PBZ*	0.114	35	26.00	25.00
	BOL**	0.086	29	7.00]	73.00
		"	21	7.50	64.50
			35	18.00	50.00
	ΦBZ***	1.00	30	0	100.00
Bradykinin 5.71×10^{-6} mg/ml	PBZ	0.114	50	50.00	0.00
	BOL (148)	0.086	42	0.00	100.00
Serotonin 6×10^{-7}	PBZ	0.114	48	52.00	0.00
	BOL (148)	0.0857	46	0.00	100.00

* PBZ Pyribenzamine

** BOL(148) dibromolysergic acid

*** ΦBZ Phenylbutazone

TABLE X

Effect of Substrate Concentration on Velocity of Formation of Dialyzable Kinin in the Presence of Sea Urchin Toxin (SUT[64-1], 2/3 SAS) at 37°C

Sea Urchin Toxin, mg/ml*	Substrate mg/ml	First Order Velocity Constants k min^{-1}	$1/[S]$	$1/k$
0.584	0.1547	0.022	6.46	44.84
"	0.1858	0.343	5.38	29.15
"	0.1981	0.469	5.05	21.32
"	0.2170	0.60	4.61	16.72
"	0.3095	1.14	3.23	8.80

* Concentrations expressed as mg protein/ml

TABLE XI

Activity of Substrates III-0 and IV-0 for the Enzyme SUT(64-2) 65 as Biological Assay of the Proteins

		ENZYME	GUINEA PIG ILEUM		RAT UTERUS
Substrate	Substrate Conc. % protein/ml	SUT(64-2)65 as mg/ml	Bradykinin (mg/ml)	Histamine (M/L)	Bradykinin (mg/ml)
III-0	0.40	0.40	1.05×10^{-4}	5.0×10^{-7}	1.11×10^{-5}
III-0	0.40	0.20	7.30×10^{-5}	4.0×10^{-7}	7.8×10^{-6}
IV-1	0.40	0.40	6.35×10^{-5}	2.8×10^{-7}	1.14×10^{-5}
IV-1	0.40	0.20	5.33×10^{-5}	2.50×10^{-7}	1.00×10^{-5}
Beef Serum	[1:10]	0.40	2.83×10^{-5}	1.73×10^{-7}	4.83×10^{-6}
		0.20	9.3×10^{-6}	5.78×10^{-8}	0.00

Incubation Period: 20 minutes
Temperature: 37°C
Inactivated: 56°C for 30 minutes

TABLE XII

Effect of Enzyme Concentration on Kinin Production from Substrate III-0
(Temp. 37°C, 20 min. inc.) SUT 65 *as*

Substrate III-0 mg/ml	SUT(64-2) 65 *as*	BK* (mg/ml) G.P. Ileum	BK mg/ml Rat Uterus
0.40	0.080	4.5×10^{-5}	5.0×10^{-6}
"	0.200	7.66×10^{-5}	9.50×10^{-6}
"	0.280	9.10×10^{-5}	1.02×10^{-5}
"	0.400	1.05×10^{-4}	1.16×10^{-5}
"	0.719	1.22×10^{-4}	1.30×10^{-5}
"	0.881	1.29×10^{-4}	1.40×10^{-5}

* Assayed as Bradykinin

Inactivated before dialysis at 56°C for 30 minutes

BK - Bradykinin

TABLE XIII

Effect of Substrate Concentration on Kinin Formation

SUT(64-2) 65 *as* (mg N/ml)	[S] III-0 (mg Protein/ml)	Incubation Time (seconds)	Bradykinin (mg/ml) ($\times 10^{-5}$)
2.96×10^{-2}	0.100	15	6.64
		30	7.11
		60	8.88
		150	9.10
		300	9.40
		600	10.00
		1200	12.30
2.96×10^{-2}	0.200	15	7.60
		30	8.00
		60	9.40
		150	10.00
		300	11.85
		600	12.50
		1200	11.60
2.96×10^{-2}	0.400	15	9.00
		30	9.25
		60	10.00
		150	11.25
		300	12.00
		600	13.20
		1200	13.60

Temperature 37°C, Inactivation 56°C for 30 minutes

TABLE XIV

Comparison of Substrate Specificities of Cohn Fractions III-0 and IV-1

SUT(64-2)65 *as* mg N/ml	[S] (mg Protein/ml)	Incubation Time	Bradykinin Equivalent ($\times 10^{-5}$)
1.27×10^{-2}	0.200 (III-0)	15	7.2
		30	7.80
		60	7.70
		150	7.50
		300	7.50
		600	8.66
		1200	8.25
1.27×10^{-2}	0.200 (IV-1)	15	1.20
		30	1.82
		60	1.90
		150	1.80
		300	1.75
		600	1.85
		1200	1.69

Temperature of Incubation 37°C
Inactivation at 56°C for 30 minutes

TABLE XV

Comparison of the Specificities of Fraction III-0 and IV-1 as Substrates Tested at Constant α-Globulin and β-Globulin Content

Experimental	Preparation	Concentration		BK equivalent G.P. Ileum	BK equivalent Rat Uterus
		Substrate	Enzyme		
β globulin constant 10%	IV-1	β = 0.04 α = 0.36	2.96×10^{-2}	1.0×10^{-4}	2.73×10^{-5}
	III-0	β = 0.04 α = 0.003		1.1×10^{-4}	0.00
α globulin constant 5%	IV-1	β = 0.0024 α = 0.021	2.96×10^{-2}	6.04×10^{-5}	2.13×10^{-6}
	III-0	β = 0.336 α = 0.021		1.15×10^{-4}	1.02×10^{-5}

APPENDIX

Investigations on Dialyzable Toxin From Hawaiian Sea Urchin

R. N. Gourley and W. A. Bonner

A sample was obtained from Professor George A. Feigen of the Physiology Department. There were 0.96 g of dialyzable material *(A)* and also 0.95 g of material, *(B)*, which was dialyzable but contained 55% of phosphate salts.

Preliminary Analysis of *(A)*

Found: C, 11.4; H, 2.4; N, 4.0; Ash 41.6%

Solubility of *(A)*

The material was not soluble in benzene, ether, methylene chloride, tetrahydrofuran, acetone, methanol or ethanol. It was almost completely soluble in water.

Thin Layer Chromatography of *(A)*

Adsorbent - Silica gel GF_{254}

The material was dissolved in a little aqueous ethanol. The spot (detected with iodine vapor or ninhydrin spray) did not move when eluted with pentane, benzene or ether. A 30% methanol - 70% ether mixture caused a very slight movement. It was found that with the following solvent systems a single spot appeared about half-way between the origin and the solvent front.

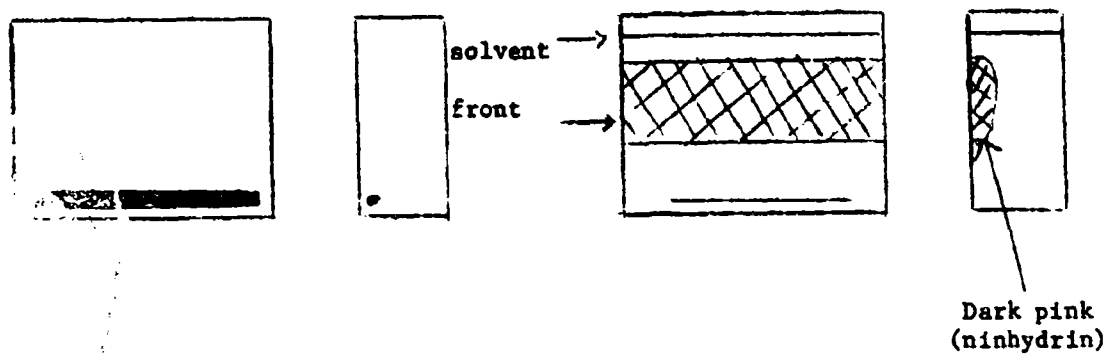
(1)	n-butanol	-	Acetic Acid	Water	Acetone
	8		2	2	3
(2)	Chloroform	-	Methanol	-17% NH_3	
	4		4	2	
(3)	Ethanol	-	Water		
	7		3		

When detected with ninhydrin, the spot was a bright pink color after the reaction with ninhydrin had taken place. This is a common method for detecting amino-acids and peptides. The above solvent systems are also used for T.L.C. on these compounds. Faint traces of material were observed both above and below the very strong spot.

Infra-red spectrum of *(A)* showed strong bands of 1650 and 11[illegible] cm^{-1} and a very broad band at 3200 cm^{-1}

Preparative T.L.C.

146 mg of *(A)* were dissolved in aqueous ethanol and prep TLC performed.
Adsorbent - silica gel GF_{254}; developer 70% EtOH - 30% H_2O



After extracting the silica gel with aqueous ethanol and evaporating the filtrates to dryness (room temperature evacuated dessicator) 76 mg of a gummy solid was obtained. This gave a small ash on burning on a glass rod in a flame. It was thought that the material contained some silica gel extracted along with the organic portion. A blank extraction of the silica gel gave 3.5 mg of solid. At present we are repeating the preparative T.L.C. on cellulose and hope that by this and by subsequent extraction of the material with aqueous ethanol to obtain a sample pure enough for analysis.

PART II

RAGWEED

INTRODUCTION

In a previous communication Meyers (1) had shown that the dialyzable material prepared from crude extracts of ragweed pollen was capable of inhibiting a variety of immunologic reactions of rabbit antisera prepared against the whole extract. The object of the present report is to present evidence that the crude antisera as well as γ-globulin fractions of such antisera can confer passive sensitivity upon intestinal and cardiac tissues of normal guinea pigs, and that the anaphylactic response to the whole antigen can be quantitatively inhibited if the dialyzable hapten is added to the system at a suitable interval before the tissues are challenged with the whole antigen

MATERIALS

Antigens: The antigens and haptens used in the immunization of rabbits as well as for challenge of sensitized tissues were prepared according to the methods described in another report (1). The particular antigens used in the present studies were preparations II, III, IV, V, and VI, all of which were obtained from defatted ragweed pollen. Preparations II and III were extracted in distilled water pH 5.0. Antigen IV was extracted with a dilute salt solution and the solution maintained at about neutrality, with stirring, by the addition of dilute NaOH. The material in the dialysate was obtained by precipitation with ethanol. Antigen V was extracted in 0.15 M phosphate buffer pH 10.8 and antigen VI was prepared in 0.15 M phosphate buffer at pH 7.6. Defatted short ragweed pollen was extracted for at least 24 hr in the above mentioned buffers or in distilled water. The insoluble portion was removed by filtration or centrifugation and the filtrate (the antigenic, non-dialyzable material) was placed in Visking bags and dialyzed against distilled water at 2°C for several 24 hour periods. The dialysates from each preparation were pooled and concentrated by pervaporation or lyophilization to give Hapten Preparations III, V, and VI.

Antisera: Antisera against the several antigens were prepared by injecting rabbits with aqueous extracts of defatted, non-dialyzable preparations. Antibody A was prepared from a pool of antisera from rabbits receiving 0.3 to 0.6 ml of 10 mg/ml of antigen intramuscularly in each hind leg. After a 10 day rest period, a course of seven to nine intravenous injections was given in which each animal received 0.5 to 1.0 ml doses of antigen at four-day intervals. Rabbits were bled 7 days after the last injection. If booster injections were required, 1.0 ml was given intravenously twice weekly for three weeks. The γ-globulin fraction was obtained by repeated precipitation in the presence of 1/3-saturated ammonium sulfate. Antibody B was prepared by immunizing rabbit No. 750 with 10.5 mg of a non-dialyzable ragweed pollen preparation, RW III. The total dose was dissolved in 3.0 ml and 1.5 ml aliquots were injected, intramuscularly, into each hind leg. The rabbit rested three months and then 0.5 ml (3.5 mg/ml) was injected intravenously twice weekly for three weeks. The rabbit was bled seven days after the last injection. The antibody concentrations of the two preparations used in these experiments were estimated at optimum proportions by the usual quantitative precipitin tests. Antibody A, the pooled γ-globulin preparation, contained 1.3 mg of specifically precipitable protein and antibody B, a crude antiserum, contained 2.30 mg of precipitable protein against the non-dialyzable Fraction IV. These preparations were shown capable of sensitizing tanned erythrocytes and producing passive cutaneous anaphylaxis.

Gut

Preparation of Tissue: Normal male guinea pigs, ranging in weight between 350 and 500 g were killed by a blow to the base of the skull. The guts were delivered into warmed Tyrode's, (2) flushed, transferred through several portions of warmed Tyrode's, and then stored in the refrigerator for 2 hours. At the end of the storage period the lower third of each intestine was cut into 3-cm strips; the tissues of all donors were combined into one pool and randomly assorted into bundles consisting of 4 strips each. The bundles of tissue were stored at room temperature in oxygenated Tyrode's for 60 min.

Sensitization of Tissue: At the completion of the last storage maneuver the tissues were transferred into a sintered glass filter tube of 60 ml capacity containing 35 ml of antibody solution prepared by diluting appropriate quantities of crude dialyzed antisera or the partially purified γ-globulin fraction with Tyrode's. Tissue bundles, removed after various periods of incubation, were washed by passage through three 30 ml portions of Tyrode's at 20°C, the lumens flushed with warm Tyrode's, and the strips positioned in 4-ml Schultz-Dale baths. Muscle contractions in response to antigens or to histamine were recorded either isometrically, by means of Statham G-7A strain gauges, or isotonically, by means of the usual kymographic method.

Challenge: All tissues were maintained in oxygenated Tyrode's solution at 37.5°C during challenge and all test antigens and haptens were made up in Tyrode's solution. The doses and concentrations of antigens used for challenge varied with the preparation and with the requirements of the experiment. In any case, the volume of antigen solution introduced into the muscle bath never exceeded 0.4 ml, the final bath volume being maintained at a constant level of 4.0 ml by the removal of Tyrode's equal in volume to the dose of antigen which was to be added. The anaphylactic reaction was allowed to develop for 10 minutes, after which the bath was flushed out and the tissue rechallenged with the same dose of antigen to confirm desensitization.

Sensitization: Each tissue was washed and then standardized with various doses of histamine. Calibration curves were constructed for each strip, as previously described by Feigen *et al.* (3, 4). The experimental results are expressed in equivalent histamine concentrations obtained by plotting the per cent of maximal response attained by each strip as a function of the histamine concentration present in the bath.

Heart

Preparation of Tissue: Pigs weighing between 250 and 300 g were heparinized (1 mg/100 g) and then killed by a blow to the base of the skull. The thoracic cage was opened and the ascending aorta was cannulated. The vena cava was transsected and the heart fed warmed Chenoweth's solution until the muscle and chambers had been cleared of blood. The cannulated heart was removed from the carcass and attached to the standpipe of an Anderson Heart Perfusion apparatus. A thread was passed through the apex of the heart in such a way that one strand could be connected to a transducer while the other was weighted with a 1.0 g load. The heart was perfused with Chenoweth's solution (5) for about 20 min while several control estimates were made of the rate, amplitude, and minute perfusion volume.

Sensitization and Challenge: Sensitization of the isolated heart was accomplished by passing 25 ml of Chenoweth's solution containing the desired antibody concentration through the coronary circuit. The effluent was collected

and reinstilled until the total volume perfused in this way was 75 ml. The organ was next 'cleared' of excess antibody by the passage of 25 ml of antibody-free Chenoweth's solution, after which it was challenged by the instillation of 25 ml of an antigen solution made up to a concentration of 0.216 mg/ml. Records of the mechanical performance were made with the aid of a strain gauge and samples of the coronary effluent were collected at 30 sec intervals in graduated centrifuge tubes.

Estimation of histamine: The samples of effluent at -20°C for the subsequent bioassay for histamine according to the methods previously described (6). At the end of the test the heart was removed, the aorta trimmed away, and the organ dried to a constant weight at 105°C. The tabulated histamine determinations and coronary flow values were normalized by dividing the raw experimental results by the dry weight of the heart.

RESULTS

Since one of the aims of the present study was to provide a quantitative estimate of the degree of inhibition by various hapten preparations it was necessary to establish the limits of antibody dilution within which the extent of the reaction could be quantitatively varied and also to arrive at a practical time of incubation for the performance of the determinations.

Limiting Dilution of Antibody

Ileal tissues of 3 guinea pigs were removed and prepared for incubation as described under the appropriate section of "Methods". Seven randomly assorted bundles of tissue were separately incubated for 1 - 3 hr with various concentrations of dialyzed antiragweed serum B, ranging between 2.3×10^{-4} to 6.0×10^{-2} mg/ml of specifically precipitable antibody protein. After incubation and washing each strip in a given bundle was tested with 0.216 mg/ml of nondialyzable antigen IV. All the guts tested reacted to that concentration of antigen after three hours of incubation. Typical Schultz-Dale reactions obtained after a 3-hour incubation with 2.3×10^{-2} mg/ml of antibody and a 1-hour incubation with 2.3×10^{-4} mg/ml of antibody are exhibited in Fig. 1. The reactions were maximal for all concentrations tested. Tissues sensitized with the higher concentrations of antibody showed a constant latent period of about 10 sec, the contractions developing rapidly and persisting throughout several washings. In contrast, the guts incubated in the presence of 2.3×10^{-4} mg/ml of antibody relaxed immediately after challenge. On the basis of these results a 1:10000 dilution of the antiserum B i.e. 2.3×10^{-4} mg of the antibody/ml, was selected as a standard concentration of antibody for the determination of the time-course of sensitization.

Time-Course

The time course of sensitization was studied by incubating portions of normal guinea pig ileum for 60, 120, and 180 min in the presence of 2.30×10^{-4} mg/ml of antibody B and, for comparative purposes, in the presence of a pooled γ-globulin preparation which contained 1.31 mg/ml of specifically precipitable antibody/ml.

Table I shows in both cases that the magnitude of sensitization increases with time of incubation and, in two instances, appears to approach a limiting value after 3 hr. Although a comparison between the two antibody systems is not strictly valid since the test antigens used were not from the same preparation it is quite obvious from Fig. 2, that antibody B produced a

comparable degree of responsiveness in a concentration several orders of magnitude lower than antibody A.

Effect of Antigen Concentration

Bundles of normal guinea pig ileum were sensitized by a three hour incubation in the presence of 3.74×10^{-2} mg/ml of pooled antiragweed γ-globulin pool "A", washed, and positioned in the 4-ml muscle chambers according to the general method. The sensitized tissues were then separately challenged in the presence of four concentrations of non-dialyzable antigen V ranging from 0.20 to 0.84 mg/ml in final concentration. In two separate instances hapten V was added to a final concentration of 5 and 8 mg/ml 15 min preceding challenge with 0.29 and 0.84 mg/ml of antigen respectively.

The results given in Table II show that the response of the sensitized tissues to increasing antigen concentration is described by a steeply rising, almost linear, function such that a 5-fold increase in response (from 2×10^{-8} to 1×10^{-7} M histamine equivalents) occurs for a slightly greater than 4-fold increase in antigen concentration. Additionally, these results demonstrate that the expected reaction to 0.29 and 0.84 mg/ml of test antigen can be blocked by 15 and 60% respectively, in the presence of appropriate concentrations of hapten added 15 min before challenge.

Effect of Hapten Concentration

Six 6-cm portions of normal guinea pig ileum were incubated for 3 hr at 37°C with 3.74×10^{-2} mg/ml of antiragweed γ-globulin-Pool A. They were then washed, and cut into 2-cm strips which were positioned and standardized with histamine according to the general technique. Following standardization with histamine appropriate quantities of hapten III were added to the bath as follows: 1.25, 1.43, and 2.86 mg/ml in Experiment I and 3.33, 5.00, and 8.00 mg/ml in Experiment II. Fifteen minutes later each tissue was challenged, in the presence of hapten, with 0.15 mg/ml of antigen III. The degrees of block were calculated with reference to the reactions given by hapten-free controls which were 1.2×10^{-7} and 2.2×10^{-7} M equivalent histamine concentrations in the first and second experiments, respectively.

The data given in Table III show that although inhibition definitely does depend on the hapten concentration present during challenge, the function rising steeply between 0 and 3.33 mg/ml of hapten, it appears to reach a plateau of 50% inhibition at hapten concentrations above 3.33 mg/ml. The effect of hapten on the change in the functional response of the tissue to challenge is illustrated by the records presented in Fig. 3. The set of kymograph records given in Fig. 3 illustrates that the concentration of antigen used for the initial challenge was sufficient to produce desensitization of the hapten-free control and that the presence of the allergen did not decrease the sensitivity of the tissue to histamine. Secondly, the presence of hapten (in the amounts used) evoked neither nonspecific nor specific reactions. Finally, the inhibitory effect of hapten is seen to be manifested both as a delay in onset and a decline in velocity of contraction, as well as in the reduction of the final magnitude of the response.

Inhibitory Power of Hapten on Degree of Sensitization

The effect of hapten on the reaction of sensitized tissues to challenge by specific antigens was tested by varying the hapten concentration when the incubation time was constant, and by determining the degree of inhibition at a constant hapten concentration when the incubation time was variable, i.e. before sensitization had become maximal.

The tissues were prepared according to the general methods given earlier. They were sensitized in the presence of 2.3×10^{-4} mg Ab B/ml for 1, 2, or 3 hr and then challenged with 0.212 mg/ml of non-dialyzable test antigen VI in the presence or absence of various hapten concentrations, as given in Table IV. In these experiments the degree of block was calculated with reference to the control.

The results given in Table IV show clearly that at a constant degree of sensitization the degree of block attainable varies with the hapten concentration present in the bath before the tissues are challenged with a constant dose of test antigen. In the present case the inhibition ranged from 17% in the presence of 0.012 mg hapten N/ml to 59% in the presence of 0.028 mg hapten. N/ml. Similarly, if the degree of sensitization were varied by different periods of incubation, the degree of block achieved by a constant hapten concentration (0.038 mg hapten N/ml) was seen to decrease from 100%, when the incubation time was 1 hr, to 56% at 3 hr when the tissue had presumably approached a maximal degree of sensitization.

Apparently the union of antibody with hapten is a firm one as repeated challenge of hapten-treated strips with the specific allergen failed to elicit a complete response in tests conducted after 30, 60, and 90 min of repeated challenge and washing cycles.

Effects on the Heart

In the present experiment six normal hearts were sensitized *in vitro* by the method of coronary perfusion described in the appropriate section under "Methods". The concentrations of antibody B used ranged from 2×10^{-5} to 2.3×10^{-3} mg/ml as specifically precipitable antibody protein. After the subsequent "clearing" maneuver, each heart was challenged with 25 ml of antigen IV, given in a final concentration of 0.216 mg/ml. Samples of the effluent were taken before challenge and then at 30 second intervals after challenge, for a period of 4.5 min. Table V, which gives the unit coronary flows and histamine release values, shows that the reduction in the perfusion volume evoked by challenge was generally greater in those hearts which received the higher antibody concentrations, although the degree of reduction was not strictly proportional to concentration. The time-courses of histamine release, given in Table V, and illustrated in Fig. 4, show that the output of histamine by the heart is a very rapid process, reaching a peak between 1 and 2 min after challenge, and that both the maximal amount of histamine produced and the rate of its release decline as the antibody concentration is reduced.

The quantitative dependence of the intensity of the reaction upon antibody concentration is illustrated in Fig. 5 by a plot of the total histamine released against the concentration of antibody used in the sensitization maneuver. The curve shows that the response to the present antiragweed preparation is easily measurable over two orders of magnitude of antibody concentration and that the lowest concentration of antibody used in these experiments, 2×10^{-5} mg/ml, is by no means at the threshold of detection.

DISCUSSION

Studies of quantitative tissue anaphylaxis are commonly based either upon the variation in strength of immunological reagents necessary to produce a constant endpoint, or upon the variation in output of a humoral agent, such as histamine, which is assumed to be released quantally (6) by certain target cells. Thus when the sensitized tissue is challenged in an optimal molecular ratio (7) the amount of humoral agent liberated becomes an indirect measure of the number of cells to which one of the specific reagents is fixed in a critical way. Although the indicator system may accurately reflect the number of successful combinations, the reaction system itself is a variable of the reagents used and of the conditions employed for sensitization and challenge.

Given a set of constant preadsorption conditions the velocity of the sensitization reaction, hence its outcome at a given time, is predictable from the antibody concentration (8, 9, 10), the ambient temperature and the time of exposure to antibody. At a constant degree of sensitization the magnitude of the reaction will also vary with the concentration, purity, and specificity of the antigen.

The effect of the nature of the antibody preparation upon the magnitude and time course of sensitization is illustrated quite clearly by comparing the quantities of histamine released after sensitization with antiragweed preparations A and B. Although the crude antiserum, preparation B, contained initially about twice as much specifically precipitable antiragweed, it produced a somewhat greater degree and velocity of sensitization at an antibody concentration about 2 orders of mangitude lower than that obtained with the partially purified γ-globulin preparation, suggesting that fractionation might have removed or inactivated a species of molecule of greater sensitizing capacity or that the test antigen contained a great deal of material that was inert - or inhibitory - to the anaphylactic response but not to the precipitation reaction.

A direct test of the effect of varying the type of antigen is afforded by comparing the results of experiment 2 with those of experiment 3 in Table I. In both cases the tissues had been sensitized with the same concentration of antibody B but had been challenged in the one case with antigen IV, and in the other with antigen VI in approximately equal concentrations. The results, as illustrated in Fig. 1, show that the rate and magnitude of sensitization appear to be greater when viewed with respect to antigen VI than when estimated with respect to antigen IV. The data show that although the onset of sensitivity in response to antigen VI was somewhat slower than to preparation IV, initially, the function appeared to accelerate in the latter case after 2 hr while in the former case it evidently had begun to reach a plateau in that interval.

Although a given quantum of pharmacological response by no means implies an invariant degree of sensitization if the frame of reference is altered, histamine release can be successfully employed to measure the effectiveness of hapten inhibition either as a function of hapten concentration or as a function of the degree of sensitization, provided that the nature of immunological reagents is not varied. In the present studies the inhibitory effects of haptens have been shown in both ways. The results obtained at constant sensitization conditions with Antibody A and antigen III show that inhibition tends to become independent of hapten concentration at a final plateau of 50%. The explanation for that may lie in the fact that the crude antigen produces antibodies against a variety of determinants besides those having specificity for the haptenic group. In the case of preparation IV tissues sensitized with antibody B showed

no such limit. The reason for this is not entirely clear - assuming that each antibody pool was composed of a mixture of sensitizing and non-sensitizing hapten-specific antibodies, both of which could compete for attachment to the number of cell receptors available, it is evident that the antibody preparation having the higher ratio of sensitizing antibodies would be the one showing a greater sensitizing power. Since the total number of antibodies required to produce a given degree of sensitization would be much lower in the case of antibody B, hapten inhibition would be much more effective as fewer of the hapten molecules would be sequestered by the anaphylactically inert antibodies.

If the degree of sensitization depends upon the number of "strongly" adsorbed antibody molecules which are critically attached to the histamine releasing cells, and if that value increases with sensitization time, as suggested from the results of King and Francis (7), it follows that the degree of inhibition by a constant, but limiting, concentration of hapten should be progressively reduced as the sensitization time is prolonged. The results obtained in Table IV when tissues incubated for 1, 2, and 3 hr were treated with 0.038 mg N/ml of hapten VI prior to challenge with non-dialyzable antigen support this view. Since the reaction between antigen and antibody required to trigger the anaphylactic response is assumed to occur at a constant ratio of unity, it follows that the degree of hapten inhibition is an indirect measure of the number of fixed antibodies reacting.

The heart is a remarkably sensitive test organ for quantitative experiments dealing with anaphylaxis by passive transfer. The amount of antibody adsorbed per unit weight of tissue and the degree of sensitization have been shown to be dependent in a simple way upon the antibody concentration in the perfusion fluid (11). Both functions attain a constant value after a single passage of antibody through the coronary circuit thus obviating delay owing to diffusion, which is characteristic of merely soaking tissues in antibody solutions. The amount of histamine released by the heart is among the greatest of all target tissues studied (12), most of it coming from the atrium which has a high total content and a large anaphylactically releasable fraction. The histaminase activity of guinea pig heart is virtually nil (13). Since the methods of chemical estimation are sensitive at least to 10^{-8} M histamine even weak anaphylactic responses can be quantitatively estimated with the degree of precision of the usual fluorometric techniques. Furthermore, since the overall pattern of response of the guinea pig heart involves an increase in rate, amplitude, and a reduction of coronary flow there is available a wide range of reliable parameters by means of which anaphylaxis may be detected even when the amount of histamine released into the effluent falls below the level of reliable analysis.

SUMMARY

(1) Passive sensitization of isolated normal guinea pig heart muscle with two different preparations of rabbit antiragweed serum failed to show a correlation between precipitating antibody and ability to sensitize.

(2) The dialyzable, small molecular weight fraction obtained from aqueous extracts of ragweed pollen inhibited the anaphylactic reaction when challenge was made with a nondialyzable fraction.

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TABLE I

Effect of Incubation Time on Reaction of *In Vitro* Sensitized Guinea Pig Ileum to Non-Dialyzable Fractions of Antigen

System		Sensitization		Challenge	
Antibody	Antigen	[AB]† mg/ml	Time (min)	[AG]† mg/ml	Histamine Equivalent moles/liter ($\times 10^{-7}$)
A*	Allergen II	3.74×10^{-2}	60	1.33	0.36
		"	120	1.33	1.60
		"	180	1.33	2.10
B**	Allergen IV	2.36×10^{-4}	60	0.216	2.72
		"	120	0.216	5.55
		"	180	0.216	7.20
B**	Allergen VI	2.36×10^{-4}	60	0.212	1.47
		"	120	0.212	4.50
		"	180	0.212	15.00

*A Antibody A was a pooled antiragweed γ-globulin which contained 1.31 mg precipitable antibody protein/ml.

**B Antibody B was a crude rabbit antiserum which contained 2.30 mg of precipitable antibody protein/ml.

† Antigen and antibody concentrations are final concentrations in the bath.

TABLE II

Effect of Varying Antigen and Hapten Concentrations on Response of *In Vitro* Sensitized Gut

Sensitization Conditions	Challenge		Inhibition		% Block
	Antigen V (mg/ml)	Histamine Equivalent moles/liter ($\times 10^{-8}$)	Hapten V (mg/ml) ($\times 10^{-8}$)	Histamine Equivalent moles/liter ($\times 10^{-8}$)	
Antibody A	0.200	2.0			
3.74×10^{-2}	0.250	2.8			
mg/ml for	0.290	3.0	5.00	2.50	16
3 hours	0.842	10.00	8.00	4.00	60

TABLE III

Effect of Varying Concentration of Hapten III on Reaction of Sensitized Gut to Constant Concentration of Antigen III

›eriment No.	Sensitization Conditions	Inhibition		Challenge		% Blocke with resp to contr
		Hapten III-2* (mg/ml)	Incubation Time with Hapten (mins)	Antigen III-2 (mg/ml)	Histamine Equivalent Moles.liter^{-1}	
I.	3.74×10^{-2} mg/ml Antibody A for 3 hours	0.00	15	0.15	1.20×10^{-7}	0
		1.25	"	0.15	9.00×10^{-8}	25.0
		1.43	"	0.15	8.60×10^{-8}	28.3
		2.86	"	0.15	8.80×10^{-8}	26.4
II.	3.74×10^{-2} mg/ml Antibody A for 3 hours	0.00	"	0.15	2.20×10^{-7}	0
		3.33	"	0.15	1.14×10^{-7}	48.2
		5.00	"	0.15	1.15×10^{-7}	47.7
		9.00	20	0.15	1.13×10^{-7}	48.6

* Added 15 minutes before challenge with antigen

TABLE IV

Effect of Varying Sensitization Time and Hapten Concentration on Response Of Gut to Antigen VI

Experimental Condition Antibody B 4.36×10^{-4} (mg/ml)	Sensitization Time (min)	Category	Hapten VI* (mg N/ml)	Antigen VI (mg/ml)	Response Hist. Equiv. (moles.liter^{-1}) ($\times 10^{-7}$) Control	Test	% Block With respect to control
Hapten Conc. Variable	180	Control	0	0.212	5.30	5.30	0
	"	Hapten	0.012	"	"	4.39	17.2
	"	"	0.019	"	"	3.65	31.0
	"	"	0.028	"	"	2.17	59.0
Incubation Time: Variable	60	Control	0.000	"	1.47	0	100.00
		Hapten	0.038	"			
	120	Control	0.000	"	4.50	1.57	65.20
		Hapten	.038	"			
	180	Control	0.000	"	15.00	6.72	56.00
		Hapten	0.038	"			

* Hapten added 15 minutes preceding challenge with antigen

TABLE V

Protocol of Anaphylactic Reactions of Guinea Pig Hearts Sensitized *In Vitro* With Various Concentrations of Rabbit Anti-ragweed Serum B

Antigen - RW IV
(0.216 mg/ml)

...ibody B (mg/ml)	Dry Heart Weight (g)	Collection Time (sec)	Coronary Flow (ml/g/min)	% of Control Flow	Histamine Released Moles/g/dry tissue
2.3×10^{-5}	0.1677	60	24.00*	100.00	0.00
		30	24.00	100.00	0.75
		"	29.80	124.00	2.23
		"	24.00	100.00	2.79
		"	21.50	89.58	3.57
		"	20.27	84.45	4.35
		"	22.65	94.38	3.37
		"	20.27	84.45	3.03
		"	24.00	100.00	2.65
		"	20.27	84.45	2.60
4.6×10^{-5}	0.1332	60	30.8*	100.00	0.00
		30	22.5	73.10	4.17
		"	19.5	63.30	5.21
		"	24.0	77.90	11.87
		"	16.5	53.60	8.28
		"	24.0	77.90	8.14
		"	22.5	73.10	7.12
		"	21.0	68.20	6.22
		"	21.0	68.20	4.13
		"	19.5	63.30	3.08
		"	18.0	58.40	
1.15×10^{-4}	0.1269	60	24.4*	100.00	0.00
		30	17.3	70.90	3.15
		"	23.6	96.00	3.78
		"	20.5	84.00	9.93
		"	18.9	77.50	13.34
		"	18.9	77.50	9.25
		"	20.5	84.00	8.59
		"	20.5	84.00	4.96
		"	18.9	77.50	4.90
		"	19.7	80.70	4.14
		"	18.9	77.50	
		"	22.1	90.60	

*Control

TABLE V (continued)

ibody B mg/ml)	Dry Heart Weight (g)	Collection Time (sec)	Coronary Flow (ml/g/min)	% of Control Flow	Histamine Released Moles/g/dry tissue
5×10^{-4}	0.1511	60	33.1*	100.00	0.00
		30	29.8	90.30	0.46
		"	23.16	70.20	4.80
		"	26.16	80.30	17.78
		"	19.90	60.30	20.13
		"	11.91	36.10	9.78
		"	10.60	32.10	7.07
		"	10.60	32.10	3.49
		"	13.20	40.00	2.09
		"	19.90	60.30	1.68
		"	21.20	64.20	
		"	19.90	60.30	
3×10^{-4}	0.1517	60	42.80*	100.00	0.00
		30	33.00	76.70	1.50
		"	10.50	24.40	13.20
		"	11.90	27.70	21.95
		"	18.50	43.00	13.91
		"	19.80	46.00	6.58
		"	21.10	49.10	3.60
		"	23.70	55.10	3.29
		"	23.70	55.10	3.18
		"	23.70	55.10	2.54
3×10^{-3}	0.1784	60	28.00*	100.00	0.00
		30	22.40	80.00	1.50
		"	13.50	48.90	10.34
		"	11.20	40.00	19.14
		"	10.70	38.20	22.07
		"	12.30	44.00	15.81
		"	13.50	48.00	8.43
		"	14.60	52.00	6.22
		"	22.40	80.00	4.64
		"	28.00	100.00	4.52

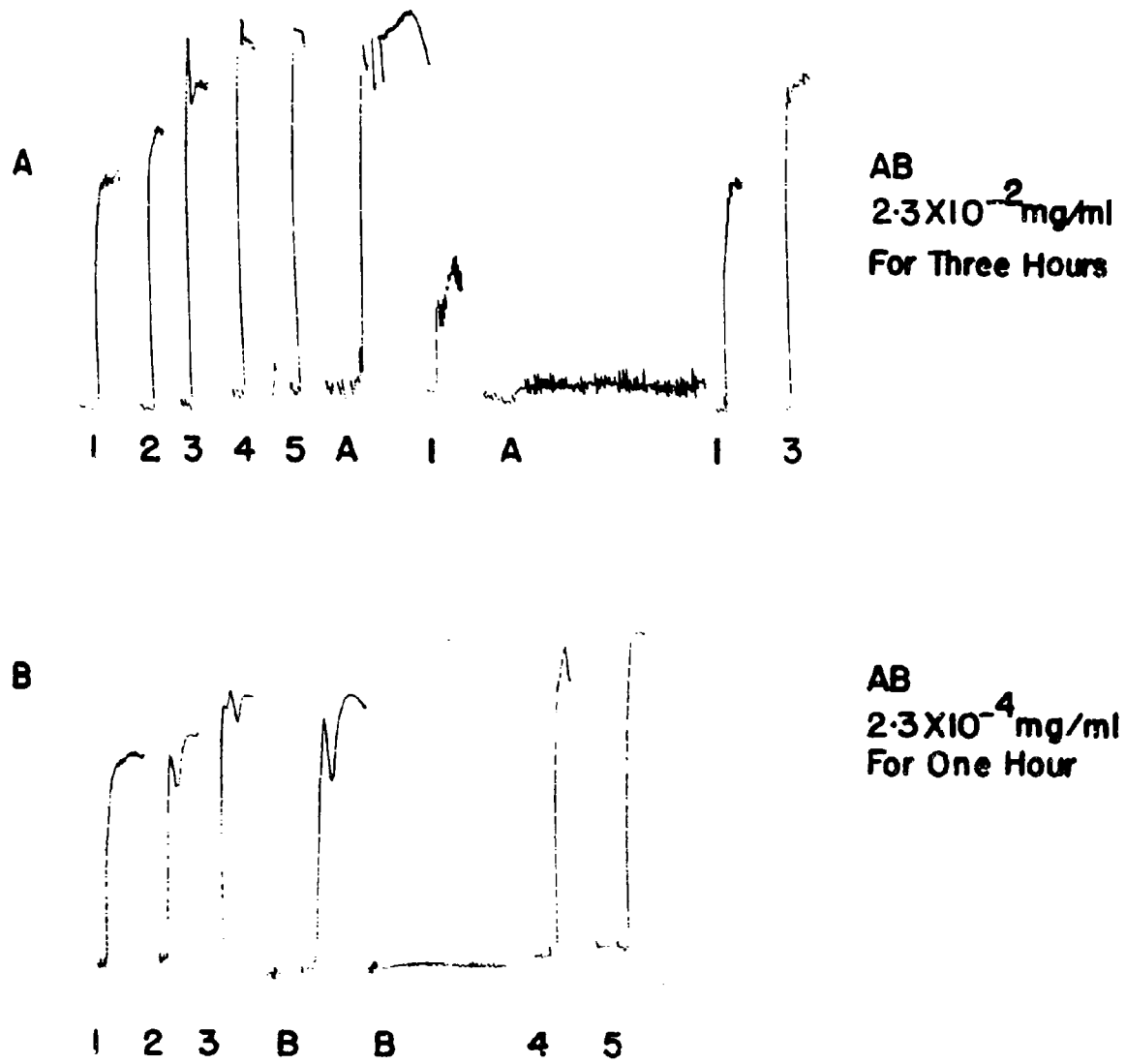

Figure 1: Effect of Antibody Concentration on Response of *In Vitro* Sensitized Intestinal Strips to Whole Antigen

A. Gut strip sensitized in the presence of 2.3×10^{-2} mg/ml of antiserum B specifically precipitable antibody for 3 hours. Standardization to histamine, as final concentrations x 10^{-8} *M* 1:5.71, 2:8.56, 3:11.4, 4:17.1, 5:22.8. Challenge and rechallenge with antigen showing desensitization to A:0.216 mg/ml Antigen IV.

B. Gut strip sensitized in the presence of 2.3×10^{-4} mg/ml of antiserum B (as above) for one hour. Standardization to histamine, as final concentrations x 10^{-7} M. 1:1.62, 2:2.1, 3:5.40, 4:8.1, 5.10.80. Challenge and rechallenge show reaction and desensitization to Antigen IV given as 0.216 mg/ml at B.

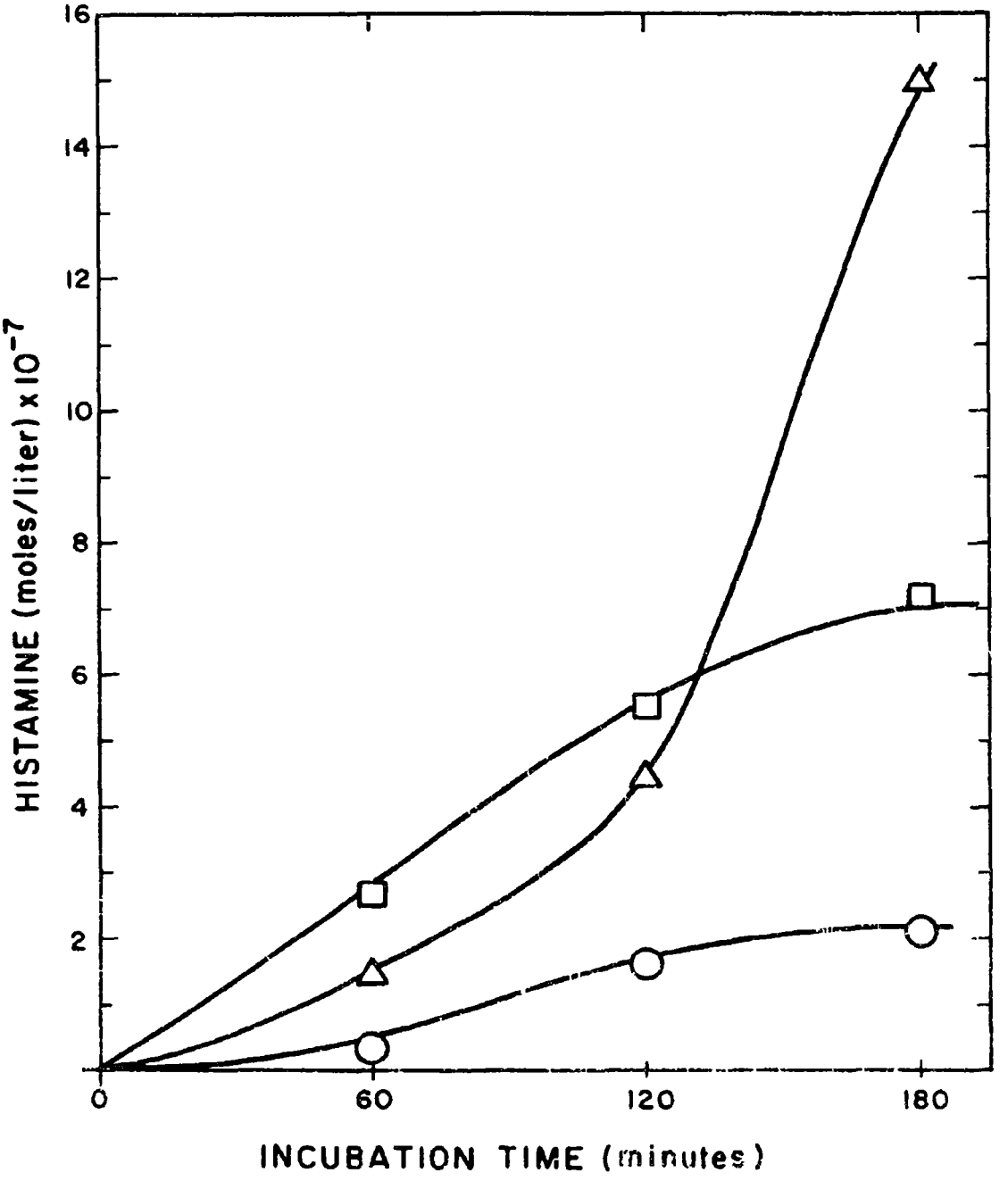

Figure 2: Time Course of Sensitization in Gut Strips as Exhibited by Various Systems

O—O Incubation with 3.74×10^{-2} mg/ml of Antibody A. Challenge with 1.33 mg/ml Antigen II.

□—■ Incubation with 2.30×10^{-4} mg/ml Antibody B. Challenge with 0.216 mg/ml Antigen IV.

△—△ Incubation with 2.30×10^{-4} mg/ml of Antibody B. Challenge with 0.216 mg/ml Antigen VI.

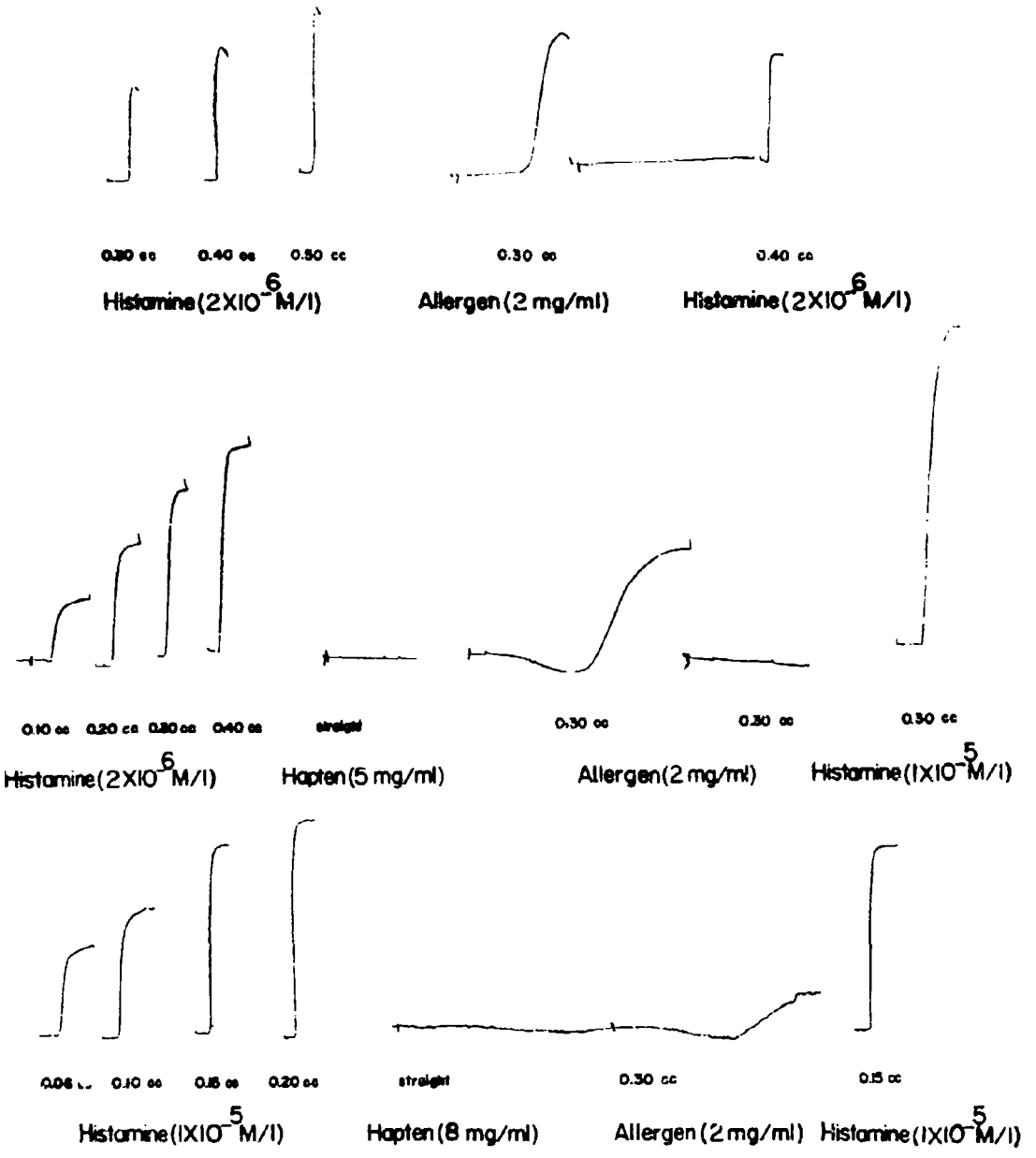

Figure 3: Inhibition of Schultz-Dale Reaction by Specific Hapten

All tissues were sensitized by incubation with 3.74×10^{-2} mg/ml Antibody A for 3 hours.

First Row: Control. Standardizations with histamine as shown. Positive reaction to Antigen III-2 followed by desensitization to same dose. Re-test with histamine, showing no decline in reactivity.

Second Row: Hapten III-2, 5 mg/ml added immediately after preliminary standardization. No reaction to hapten alone. Reaction to Antigen in presence of hapten is of decreased magnitude and velocity with respect to control.

Third Row: Hapten III-2, 8 mg/ml. This set shows very weak response to Antigen III-2 in the presence of hapten. Sensitivity to histamine not impaired by inhibition and challenge procedures.

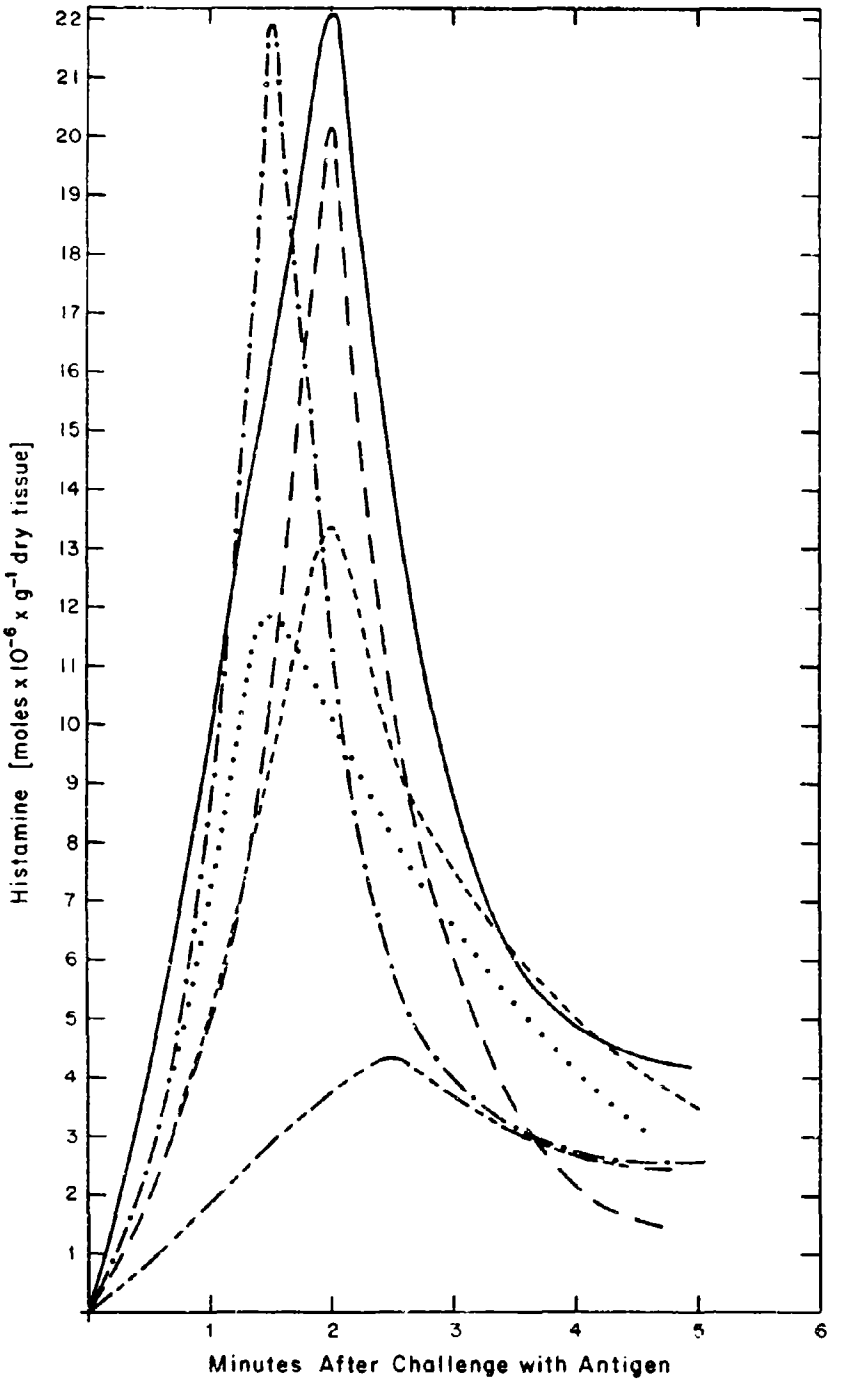

Figure 4: Time Course of Histamine Release From Hearts Sensitized with Various Concentrations of Antibody B and Challenged with A Constant Dose of Antigen IV

Antibody B:

- 2.3 x 10^{-5} mg/ml
- 4.6 x 10^{-5} mg/ml
- 1.2 x 10^{-4} mg/ml
- 1.6 x 10^{-4} mg/ml
- 2.3 x 10^{-4} mg/ml
- 2.3 x 10^{-3} mg/ml

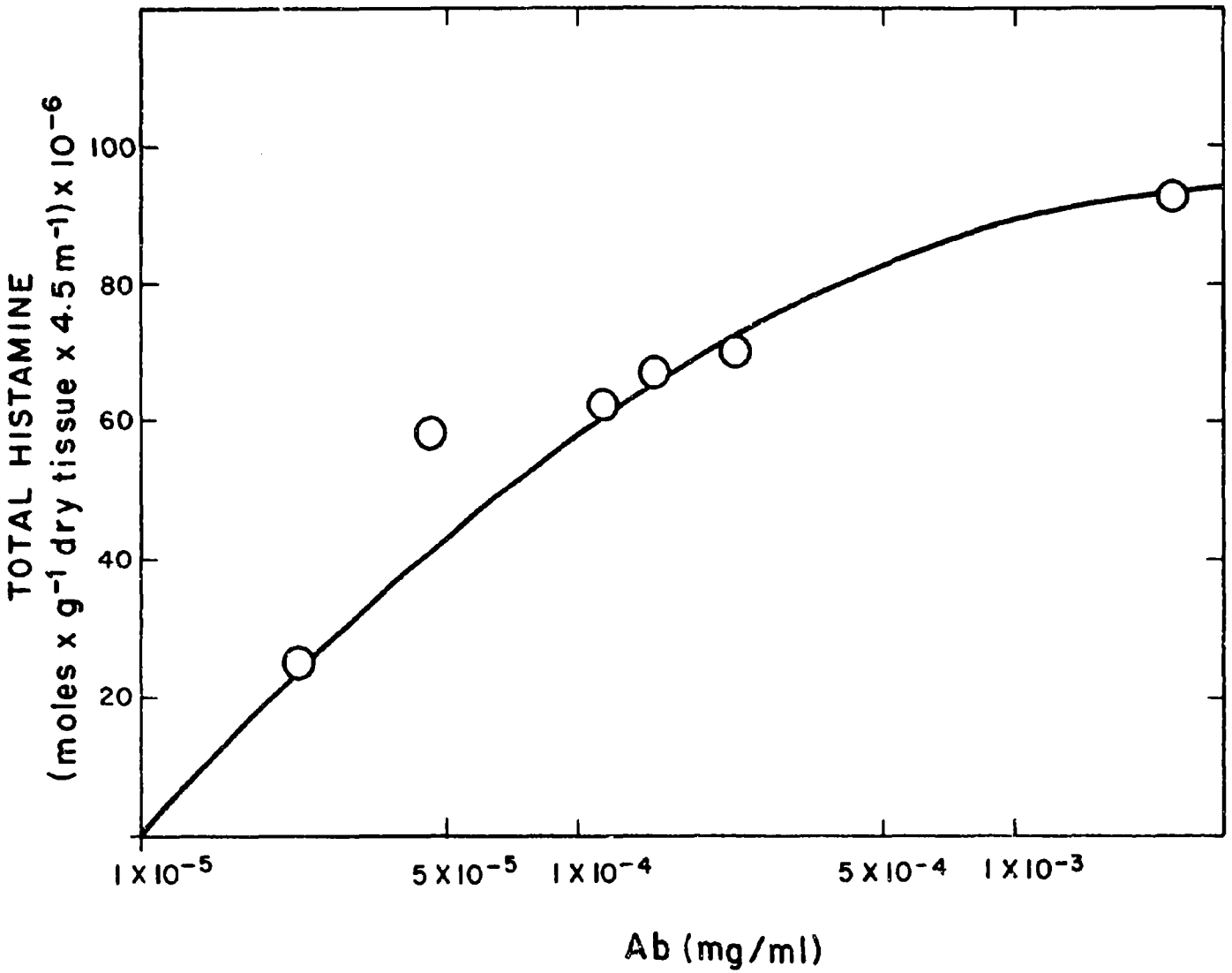

Figure 5: Variation of Total Histamine Release During Cardiac Anaphylaxis as a Function of Sensitizing Antibody B Concentration

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13 ABSTRACT Research under the purview of Contract ONR 225(46) has two general directions. 1) The study of the mode of action of protein toxins, and 2) the study of the mechanism of anaphylaxis, particularly with respect to the process of sensitization. Several distinct problems are being prosecuted under each category. Tetanus: The non-spasmogenic factor in tetanus toxin produces a stimulation of miniature end plate potentials in skeletal muscles. Chromatography on Sephadex G-200 gave 3 major peaks. The yields of repeated runs were separately pooled. No appreciable MEPP activity was found in the first 2 peaks but appeared to be concentrated in Peak III, which had no lethal activity. This peak had only one antigenic determinant and gave a Svedberg coefficient of 2. All of the lethal toxicity of the parent was quantitatively recovered in Peaks I and II which contained 5S and 4S molecules, respectively. The MEPP material accounted for only 7% of the total protein of the starting material. Streptolysin: Streptolysin O gives a double insult to the heart, one of these is reversible, and is due largely to the output of acetylcholine, while the other appears to be more severe and leads to the release of K^{42}. Sea Urchin Toxin: The crude pedicellarial toxin of *Tripneustes gratilla* obtained from 1,000 specimens in 1966 and 2,000 specimens during 1967 was variously fractionated, and the fractions further purified by chromatography on Sephadex. The enzymological properties of these materials were studied. The kinetics of the reaction system with respect to whole plasma and to its pseudoglobulin fractions are of a complex order owing to (1) "natural" kinin formation of the substrate, (2) to the inactivation of the reaction product (and of synthetic bradykinin) by the toxin, and (3) to the existence of more than one enzyme in the "purified" material. Present studies show that

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one of the substrates is α_2-globulin.

ANAPHYLAXIS

Ragweed: Rabbit anti-ragweed conferred passive sensitivity on cardiac and intestinal tissues of the guinea pig. The degree of sensitization was shown to depend on the [AB] in the bulk phase. For a constant degree of sensitization the response to the whole antigen was quantitatively inhibited by a dialyzable ragweed hapten. Penicillin: Positive anaphylactic reactions were obtained to penicilloyl poly-lysine in isolated guinea pig tissues passively sensitized with rabbit antibodies. The reaction could be quantitatively blocked by ε-aminocaproic acid and by the penicilloyl derivative of the condensation product between DAB (1, 4-diaminobutane) and fluorescein isothyocyanate. Fc Fragments: Quantitative reversed passive sensitization with Fc fragments of rabbit γ-globulin was demonstrated.

14. KEY WORDS	LINK A		LINK B		LINK C	
	ROLE	WT	ROLE	WT	ROLE	WT
Tetanus Toxin, non-spasmogenic factor, properties						
Sea Urchin Toxin, preparation, purification, enzymatic properties						
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